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14. ABSTRACT
Tumor metastasis is a complex and often fatal complication of most cancers. One of the biggest challenges to treatment is that prior to diagnosis or during treatment tumor cells can disseminate and remain dormant in distant tissue sites. These cells can become proliferative and lead to metastatic disease late after completion of therapy. The biology of this outbreak of dormant tumor cells that leads to relapsed metastatic disease is the major focus of this grant. Using a fibrosis model of tumor dormancy we have determined the break in dormancy is dependent on collagen and other fibrotic extracellular matrix components for the induction of a proliferative state in these dormant D2.0R breast cancer cell lines. Performing gene expression array on these dormant D2.0R cells exposed to collagen to induce a break from dormancy compared to dormant D2.0R cells revealed a set of genes that overlap with published dormancy gene sets. We also have performed immunophenotyping of the microenvironment of proliferating D2.0R cells in the fibrosis model of tumor dormancy and have identified an expansion of mesenchymal stem cells coincident with this metastatic outgrowth. We are now performing studies to analyze the key chemokine/cytokines released from the tumor cells transitioning from a dormant to proliferative state that may recruit these mesenchymal cells. We then plan to delve deeper into the crosstalk between these mesenchymal cell populations and the tumor cells to delineate the molecular pathways, which inform this complex biology. We plan to use both our *in vivo* and *in vitro* models with conditional gene deletion in the specific cell populations to determine the functional role of each key molecular component in the break from tumor dormancy. We anticipate these findings can identify potential therapeutic approaches to inhibit metastatic progression.

15. SUBJECT TERMS Breast cancer; dormancy; tumor recurrence; stroma; cytokines; chemokines; mesenchymal stem cells; hematologic stem cells; metastasis; quiescence; animal models; fibrosis; basement membrane extract; 3D culture

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1. INTRODUCTION:

Despite successful treatment of the primary tumor and years of disease free survival in breast cancer patients, recurrent metastatic disease is a major cause of morbidity and mortality. Accumulating evidence strongly suggests that the extended period of tumor latency is due to the survival of disseminated tumor cells that exist in a dormant state. The purpose of this research is to identify mechanisms of tumor cell dormancy using novel *in vitro* and *in vivo* models of mammary cancer dormancy that we have developed. This work seeks to identify chemokines/cytokines that are involved in regulating the switch of dormant cells into a proliferate state. In addition, this study also seeks to identify interactions between dormant tumor cells and stromal cells that contribute to the dormant-to-proliferative switch. Identifying these mechanisms that regulate dormancy or the dormant-to-proliferative switch will potentially provide molecular targets that could be exploited to prevent the proliferative outbreak of dormant tumor cells or perhaps enhance this proliferative switch to more effectively kill these disseminated tumor cells, thus preventing disease progression.

2. KEYWORDS:

Breast cancer; dormancy; tumor recurrence; stroma; cytokines; chemokines; mesenchymal stem cells; hematologic stem cells; metastasis; quiescence; animal models; fibrosis; basement membrane extract; 3D culture

3. ACCOMPLISHMENTS:

What were the major goals and objectives of the project?

The major goals of this project are1) to identify chemokines/cytokines that are involved in cross-talk between dormant tumor cells and stromal cells that influence the dormant-to-proliferative switch and that influence the tumor microenvironment, and may recruit MSCs and HCs to the dormant cell niche to enhance proliferation of the dormant cells; and 2) To determine the contribution of HCs, MSCs and resident stromal cells in activating the dormant-to-proliferative switch and metastasis using established *in vivo* models of mammary tumor cell dormancy.3) To target cytokines/ chemokines based on a candidate approach and those identified in Goals 1 and 2 to prevent the proliferative switch in dormant tumor cells.

What was accomplished under these goals?

The Green and Kaplan laboratories have been working collaboratively with the work divided based on each labs' expertise to accomplish the stated tasks in the submitted SOW. We meet regularly to review data and to plan experiments. We have made considerable progress on this work.

Aim 1: **To identify cytokines/chemokines produced by dormant tumor cells triggered to proliferate in vitro and in vivo that influence the tumor microenvironment and may recruit MSCs and HCs.**

Tumor-secreted cytokines during dormant to proliferative switch using an in vitro 3D culture model.

We have previously demonstrated that D2.0R breast cancer cells cultured in matrigel ("3D culture") alone remain dormant ("dormant D2.0R") and proliferate when cultured in matrigel supplemented with collagen type-1 ("proliferative D2.0R") (Barkan, Cancer Research, *2008*, 68(15)). In order to determine what cytokines are secreted by D2.0R cells during this dormant to proliferative switch, we collected the culture supernatants from our 3D culture model for use on a protein-based cytokine array. We identified a panel of cytokines that were differentially secreted by proliferative D2.0R cells (matrigel + collagen) compared to dormant D2.0R cells (matrigel alone). The most differentially secreted cytokine in this assay was IL6. We next confirmed that IL6 is elevated under these *in vitro* conditions using an ELISA-based system (Fig 1). We are now investigating the potential functional role of IL6 in the dormant-to-proliferative switch with the use of neutralizing antibodies to IL6 *in vitro*, and plan to investigate the role of IL6 in mobilization or maturation of MSCs to enhance the dormant-to-proliferative switch.

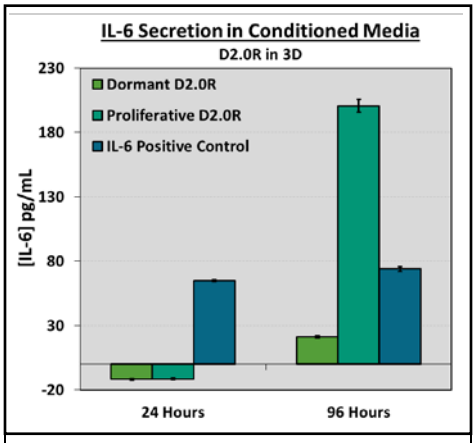


Figure 1. *ELISA validation of IL-6 Expression.* D2.0R cells in the proliferative state secrete 10-fold more IL-6 compared to the dormant state.

Gene expression signature of dormant to proliferative switch.

We have characterized gene expression of proliferative (collagen + matrigel) vs. dormant (matrigel alone) D2.0R cells in 3D culture. This work was performed using the Affymatrix Exon Array ST 1.0 with ~190,000 probes covering ~ 23 K transcript IDs, and 16 K gene symbol annotations. ANOVA was used with FDR p value < 0.05% and fold-change +/- 2 to identify genes whose expression was most significantly changed. We have analyzed the microarray data and compared our differentially expressed gene sets to a published data set of tumor cell dormancy. We identified a set of 14 genes associated with the dormant-to-proliferative switch which corresponds to a previous

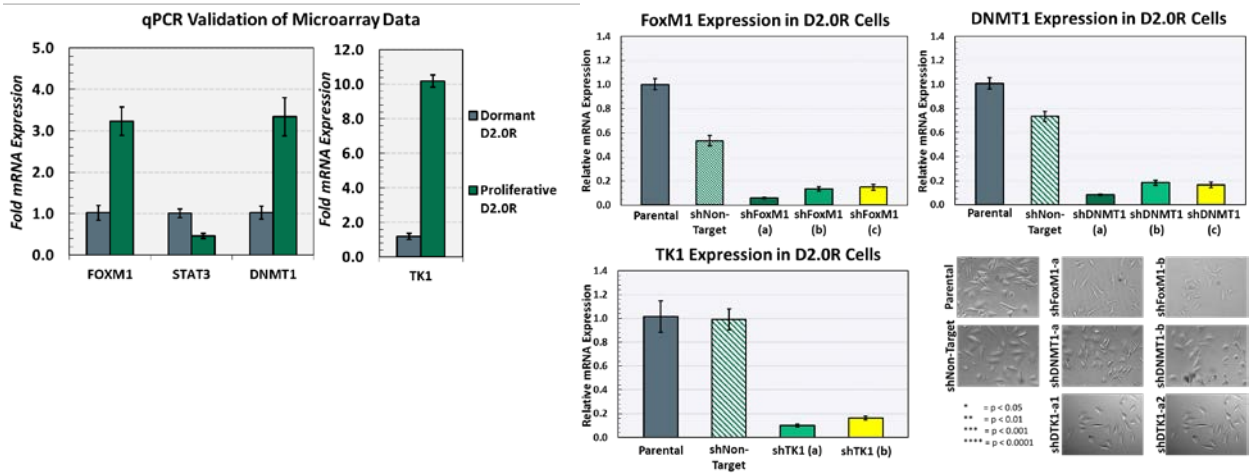
study that described a dormancy signature derived from analyses of *in vitro* and patient tumor samples (Kim et al, PlosOne, 2012;7(4):e35569). Given that since these genes have been identified in two different experimental systems for dormancy, these genes are high priority candidates for further evaluation in future studies.

Candidate genes from our microarray analysis (Fig 2a) were further validated by quantitative real-time PCR, including FoxM1, STAT3, DNMT1, and TK1 (Fig 2b). To determine if the dormant-to-proliferative switch in our *in vitro* 3D system is dependent on FOXM1, DNMT1 or TK1, we have established D2.0R cell lines with shRNA-mediated stable knock-down of each of these three genes. Their knock-down efficiency was validated via q-rtPCR (Fig 2c) prior to performing functional assays. Preliminary experiments utilizing these stable cell lines in our 3D model system are on-going. In parallel, we are utilizing a DNMT1-specific small molecule inhibitor in our 3D system to complement our shRNA-mediated stable knock-down approach.

A

D2.0R in Proliferative-Outbreak vs. Dormant States (24 Hours)

Gene Name	Abbr.	Fold Change	P-Value	Function
Budding Uninhibited by Benzimidazoles 1 homolog	BUB1	3.27	0.0029	Centromere assembly
Budding Uninhibited by Benzimidazoles 1 homolog, beta	BUB1B	2.63	0.0069	Cell Cycle, spindle assembly
Cyclin-Dependent Kinase Inhibitor 3	CDKN3	2.42	0.0135	Cell cycle
DNA Methyltransferase (cytosine-5)	DNMT1	1.84	0.0001	DNA methylation
Fos-like Antigen 1	FOSL1	1.29	0.0053	Differentiation, proliferation
Forkhead Box M1	FOXM1	2.96	0.0009	Cell Cycle, DNA damage
Phosphatidylinositol 3-Kinase, Catalytic, beta polypeptide	PIK3CB	1.19	0.0234	Motility, proliferation, and cell survival
Thymidine Kinase 1	TK1	3.45	0.0009	Biomarker for recurrence
Cathespsin D	CTSD	-1.48	0.0044	Intracellular breakdown of proteins
Signal Transducer and Activator of Transcription 3	STAT3	-1.5	0.0005	Cell growth, apoptosis
Actavin A receptor, type 1	ACVR1	-1.28	0.027	Cell growth?
Basic Helix-Loop-Helix Family, member e41	BHLHE41	-1.32	0.0518	Cell differentiation
Collagen, type 5, alpha 5	COL4A5	-1.39	0.0046	Basement Membrane Component
Tropomyosin 1, alpha	TPM1	-1.14	0.0436	Cytoskeleton stability, muscle contraction



B **C**

Figure 2. A) *Summary of genes implicated in tumor cell dormancy*. D2.0R cells were cultured in BME (dormant) or BME+Col-1 (proliferative) conditions for 24 hours, and gene expression was characterized by microarray. **B)** *Validation of tumor cell dormancy genes by RT-qPCR*. Genes FoxM1, STAT3, DNMT1, and TK1 showed differential gene expression by qPCR, which matches microarray data. **C)** *Expression of shRNA targeting dormancy genes shows stable knockdown of genes in D2.0R cells*. We see a stable decrease in expression of tumor cell dormancy genes by 80-90% of parental expression levels. There are no appreciable morphological differences amongst cell lines.

Future experiments will assess the functional role of these three genes in our *in vivo* dormant-to-proliferative switch model. In particular, our finding DNMT1 is elevated is of particular interest has it has been associated with STAT3 transcriptional regulation that is aberrantly expressed in breast cancer and in particular breast cancer stem cells (refs).

Aim 2: **To determine the contribution of HCs, MSCs and resident stromal cells in activating the dormant-to-proliferative switch and metastasis using established in vivo models of mammary tumor cell dormancy.**

Characterizing the HC, MSC and resident stromal cell populations during the dormant to proliferative switch in vivo.

Previously, we have demonstrated that D2.0R breast cancer cells that are intravenously (IV) injected into naïve mice arrive in the lungs, yet remain dormant as single cells. In contrast, D2.0R cells proliferate when IV injected into mice that have TGFß-induced fibrosis in the lungs (Barkan, Cancer Research, *2008*, 68(15)). In order to characterize the cellular players within the microenvironment that may contribute to the dormant-to-proliferative switch, we have performed two large *in vivo* experiments and performed extensive immunophenotyping in the lungs of mice with or without TGFβ induced fibrosis and with or without D2.0R tumor cells. We determined that MSC levels are decreased in fibrotic lungs prior to the introduction of tumor cells, potentially due to MSC differentiation into activated fibroblasts (Fig 3a). Interestingly, we observed expansion of the MSC population in fibrotic lungs after tumor cell injection.

To validate our new findings that MSC levels are altered with fibrosis and the introduction of tumor cells, we have also performed a Colony Forming Unit-Fibroblast (CFU-F) assay to functionally assess the number of MSCs within the lungs of fibrotic and non-fibrotic tumor bearing and non tumor bearing mice (Fig 3b). We saw a significant increase in type-1, fast replicating colonies from fibrotic, non-tumor-bearing mice compared to non-fibrotic, non-tumor-bearing mice. These type-1 colonies characterize differentiating MSCs and may recapitulate the decreasing MSC population shown at weeks 3 and 6 by flow cytometry (Fig 3a) that may be differentiating into activated fibroblasts. Although we did not see any change in type-2, slow replicating colonies, which characterize the most stem-like MSCs we did observe a significant increase in type-1D, dense fast replicating colonies from tumor-bearing mice with or without fibrosis. We hypothesize that these colonies may represent activated fibroblasts, suggesting that tumor specific factors potentiate or activate MSCs to become myofibroblasts. Given the limited data on mesenchymal stem cell differentiation into different lineages including fibroblasts these investigations into the role of organ and bone marrow-derived mesenchymal stem cells and fibroblasts during fibrosis and disseminated tumor cell growth are fruitful and much need of further investigation. These studies can potentially provide new insights into this critical stromal cell biology and new therapeutic strategies for fibrosis and cancer progression.

In addition to mesenchymal cell investigations wee did extensive analysis of hematopoietic stem cells and their progeny including myeloid cell populations and were unable to characterize these populations due to the mixed background of CD1 nude mice that can alter the baseline levels of these immune cells (Fig 3c). We therefore are using the alternate approach of colony forming assays to assess hematopoietic stem and progenitor cell functionally within the fibrotic and non-fibrotic lung.

Bone marrow transplantation of RFP-labeled bone marrow cells into recipient nude mice, induction of fibrosis and injection of dormant D20R cells and imaging/flow cytometry of lungs to characterize MSC and HC infiltration.

We proposed to acquire a breeding pair of B6.Cg-Tg(CAG-mRFP1)1F1Hadj/J mice from Jackson labs. Mice were to be crossed with CD1nu/nu mice for 5 – 10 generations to generate CD1nu/nu-Tg(CAG-mRFP1) mice for use in bone marrow transplant studies. Our flow cytometric studies using the *in vivo* tumor dormancy fibrosis model revealed that the mice are of different background (Fig 3c), thus we have postponed the transplantation experiments until after we obtain further data as to which populations of cells are altered in this setting and could be contributing to the dormant to proliferative switch. We cannot rely on immunophenotyping these mice given the mixed background. Consequently, we will use colony forming unit assays to help answer the question of hematopoietic cells. The transplant studies will also be used to understand better the origins of the mesenchymal cell populations we found altered in the lung of fibrotic, tumor-bearing mice compared to non-fibrotic, non-tumor-bearing mice. Similarly, we

will postpone experiments using AMD3100 to mobilize bone marrow derived cells in the context of our *in vivo* tumor dormancy fibrosis model. These experiments will be performed in the future as outlined in the SOW after we have validated our preliminary results regarding changes in MSC infiltration in fibrotic, tumor-bearing lungs compared to non-fibrotic, non-tumor bearing lungs.

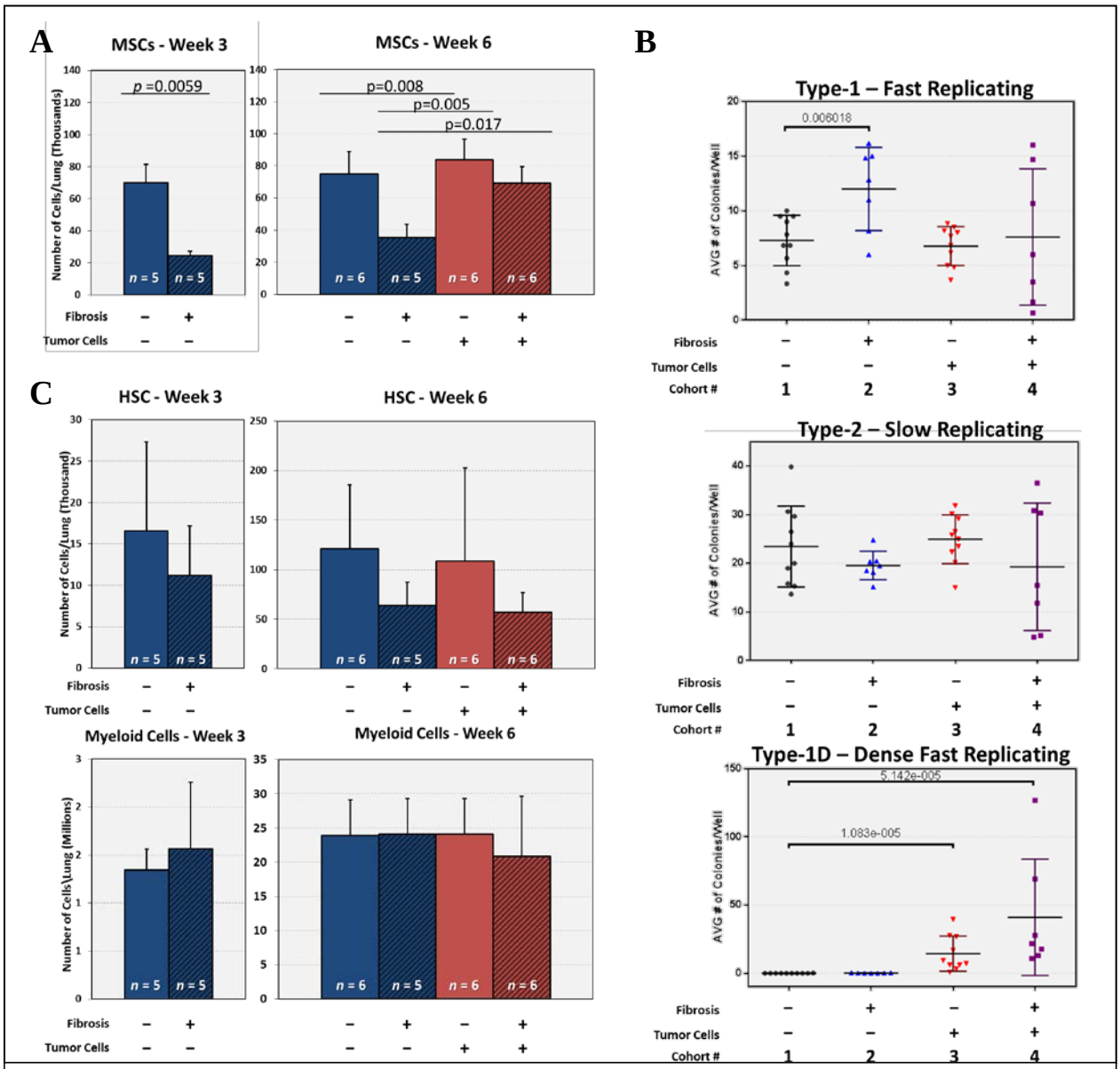


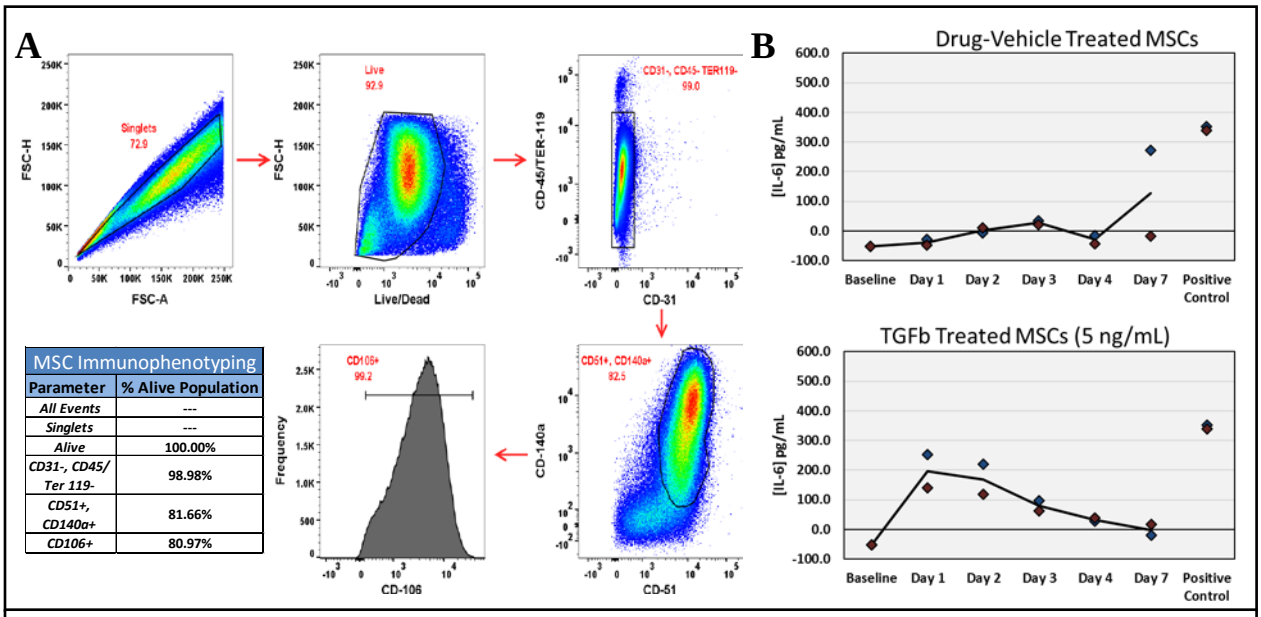
Figure 3. A) *Mesenchymal stem cell (MSC) population numbers by flow cytometry***.** MSC populations decreased in response to fibrosis and absence of tumor cells, compared to non-fibrotic, non-tumor-bearing mice at weeks 3 and 6. The presence of tumor cells in the context of fibrosis ameliorated this decrease in MSCs. **B)** *Characterization of MSCs by Colony Forming Unit – Fibroblast assay*. Fibrotic, non-tumor-bearing mice show a modest increase in Type-1 colonies as compared to non-fibrotic, non-tumor-bearing mice. There were no significant differences in type-2 colonies. There was a significant increase in Type-1D colonies in tumor-bearing mice compared to non-tumor-bearing, which was further increased in the presence of fibrosis. **C)** *Hematopoietic Stem cells (HSCs) and Myeloid Cell population numbers by flow cytometry*. No statistical difference amongst groups due to intra-group variability.

Aim 3: To target cytokines/ chemokines based on a candidate approach and those identified in Goals 1 and 2 to prevent the proliferative switch in dormant tumor cells.

Determination of inhibiting MSC and HC functions in initiating dormant-to-proliferative switch.

Given our findings that MSC levels are decreased in the lungs of non-tumor-bearing mice in response to fibrosis, but are elevated when tumor is introduced, we are focusing our functional *in vitro* studies on the MSC population. We have acquired an MSC cell line from Cyagen and immunophenotyped these cells (Fig. 4a). We have demonstrated that, like D2.0R cells that are cultured in matrigel supplemented by collagen (Fig. 1), MSCs secrete IL6 when cultured in TGFß-containing medium (Fig. 4b). This finding re-enforces that IL-6 might be a key signaling axis in the dormant-to-proliferative switch, and is a key target for our proposed inhibition studies *in vivo*. To determine the functional role of IL6 signaling, we are currently utilizing recombinant IL6 protein and siRNA targeting IL6 in our *in*

vitro culture systems for MSCs, D2.0R breast cancer cells, and in combination. Concurrently, we are using culture supernatants from MSCs +/- TGFß, and D2.0Rs in matrigel +/- collagen to assess whether IL6 is a key signaling axis for cross-talk between these two populations to promote the dormant-to-proliferative switch by D2.0R cells. Consequently, we hypothesize that IL-6 mediates signaling between these two populations to promote the dormant-to-proliferative switch of D2.0R cells.



MSC Immunophenotyping	
Parameter	**% Alive Population**
All Events	---
Singlets	---
Alive	100.00%
CD31-, CD45/ Ter 119-	98.98%
CD51+, CD140a+	81.66%
CD106+	80.97%

Figure 4. A) *Characterization of MSCs.* MSCs were immunophenotyped by flow cytometry using cell surface markers described above. Over 80% of live cells were positive for MSC surface markers. **B)** *ELISA of IL-6 secretion by MSCs treated with drug vehicle or TGFb*. MSCs treated with TGFβ show increased secretion of IL-6 over several days as compared to drug vehicle-treated MSCs.

Inhibition of IL6 using the in vivo model of fibrosis-induced dormant-to- proliferative switch.

We are currently targeting IL6 in our *in vivo* fibrosis model of the dormant-to-proliferative switch to assess proliferative outgrowth of D2.0R cells in the context of neutralizing antibody-based IL6 inhibition. Subsequent experiments will focus on the contribution of MSCs to the dormant-to-proliferative switch under the context of IL6 inhibition.

<u>What opportunities for training and professional development did the project provide?</u>

This work supports the training of two post-baccalaureate students in the Green and Kaplan labs as well as training of the lab in the continued growing area of tumor cell dormancy, cytokine/chemokine analyses, gene expression profiling and characterization of stromal cell components that may play critical roles in the dormant-to-proliferative switch. Ryan Nini presented a subset of this work at the annual AACR meeting in New Orleans this year.

<u>How were the results disseminated to communities of interest?</u>

We will publish our findings once these studies have been completed. The Green and Kaplan groups meet regularly to share data and discuss experimental designs of future experiments.

<u>What do you plan to do during the next reporting period to accomplish the goals and objectives?</u>

IL6 targeting experiments in vitro and in vivo along with DNMT1 inhibition genetically and with a small molecular inhibitor will provide functional insight into these discoveries made regarding the biology of MSC contribution to myofibroblasts and the dormant-to-proliferative switch. These investigations will be part of the larger body of work presented to be published in the coming year.

4. IMPACT:

- **the development of the principal discipline(s) of the project;**
  Nothing to report
- **other disciplines;**
  Nothing to report
- **technology transfer; or**
  Nothing to report
- **society beyond science and technology.**
  Nothing to report

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change.**
  Nothing to report.
- **Actual or anticipated problems or delays and actions or plans to resolve them.**
- **Changes that have a significant impact on expenditures.**
  Nothing to report
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.**
  Nothing to report.

6. PRODUCTS:

- **publications, conference papers, and presentations;**
  Nothing to report
- **website(s) or other Internet site(s);**
  Nothing to report
- **technologies or techniques;**
  Nothing to report
- **inventions, patent applications, and/or licenses; and**
  Nothing to report
- **other products.**
  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Provide the following information on participants:

Name:	Jeffrey E. Green, M.D.
Project Role:	Initiating P.I.
Nearest person month worked:	4
Contribution to Project:	Designs and oversees experimental progress; interprets data.
Funding Support:	The Center for Cancer Research, NCI, Bethesda, MD.

Name:	Rosandra Kaplan, M.D.
Project Role:	Co-P.I.
Nearest person month worked:	4
Contribution to Project:	Designs and oversees experimental progress; interprets data.
Funding Support:	The Center for Cancer Research, NCI, Bethesda, MD.

Name:	Ryan Nini, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	24
Contribution to Project:	Performs *in vitro* and *in vivo* experiments related to dormancy; Performs cytokine analyses *in vitro and in vivo;* analyzes gene expression profiling data; performs FACS analyses of *in vitro* experiments designs experiments; interprets data.

Name:	Lara El Touny, Ph.D.
Project Role:	Post-doctoral Fellow
Nearest person month worked:	2
Contribution to Project:	Designs and oversees experimental progress; interprets data.

Name:	Caitlin Reid, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	15
Contribution to Project:	Performs FACS analyses of *in vivo* experiments.

Name:	Amber Giles, Ph.D.

Project Role:	Post-doctoral Fellow
Nearest person month worked:	3
Contribution to Project:	Performs FACS analyses of *in vivo* experiments.

Name:	Meera Murgai, Ph.D.
Project Role:	Post-doctoral fellow
Nearest person month worked:	4
Contribution to Project:	Performs cytokine analyses *in vitro and in vivo*. Designs and oversees experimental progress; interprets data.

Name:	Kush V Bhatt, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	1
Contribution to Project:	Continuing previously conducted FACs analyses of *in vivo* experiments

- **Has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  Nothing to report
- **What other organizations have been involved as partners?**
  Nothing to report